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Studies on the Binding of RNA Polymerase to Polynucleotides

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A simple, rapid method for detecting the binding of RNA polymerase to native DNA has been developed. Whereas native DNA and RNA polymerase, separately, pass through Millipore membrane filters, a complex of the two is quantitatively retained. With this technique the stoichiometry and some properties of the complex between native T7 DNA and RNA polymerase have been measured; we estimate that there are 35 to 70 binding sites for RNA polymerase per mole of T7 DNA.

Other polynucleotides block the formation of the T7 DNA–RNA polymerase complex if added to RNA polymerase before but not after the T7 DNA. Measurements of the effect of polynucleotide concentration on the extent of inhibition of complex formation between T7 DNA and RNA polymerase suggest that the number of binding sites of RNA polymerase to nucleic acids depends upon the primary and secondary structure of the polynucleotides.

1. Introduction

A still unanswered but crucial question regarding RNA synthesis is how and where RNA polymerase initiates transcription on the DNA. If specific regions are initiator sites, what distinguishes them from the remainder of the molecule and how does the enzyme recognize and bind to them? One recent clue is that RNA synthesis *in vitro* appears to start with a purine (Bremer, Konrad, Gaines & Stent, 1965; Maitra, Novogrodsky, Baltimore & Hurwitz, 1965; Maitra & Hurwitz, 1965) suggesting a pyrimidine in the template as part of an initiator site.

There is considerable evidence (Fox, Gumport & Weiss, 1965; Berg, Kornberg, Fancher & Dieckmann, 1965; Kadoya *et al.*, 1964; Crawford, Crawford, Richardson & Slayter, 1965; that RNA polymerase and DNA form relatively non-dissociable complexes even in the absence of RNA synthesis. Several investigations have established that polynucleotides compete with each other for available enzyme and this is manifested by inhibition of transcription (Krakow & Ochoa, 1963; Wood & Berg, 1964; Fox, Robinson, Haselkorn & Weiss, 1964; Fox *et al.*, 1965; Berg *et al.*, 1965). The fact that inhibition depends upon the order of mixing of enzyme, DNA template and inhibitor polynucleotide (Fox *et al.*, 1965; Berg *et al.*, 1965; Chamberlin, personal communication) indicates that the binding of RNA polymerase to DNA is not readily dissociable (Wood & Berg, 1964). Recent experiments showed that RNA polymerase binds strongly at or near the ends of

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helical DNA and thereby blocks enzymes which require free ends or which initiate their action from the ends (Berg *et al.*, 1965). These studies did not eliminate the likelihood that there were non-terminal sites in native DNA for attachment of RNA polymerase. Moreover, there was no information about how binding of enzyme to DNA was influenced by the secondary structure of the DNA. Conceivably, studies of this binding and its parameters could help determine whether discrete RNA polymerase-binding sites exist in DNA and if these sites are where RNA synthesis begins.

In the course of this investigation we discovered a rapid and simple method for detecting the binding of RNA polymerase to native DNA. Whereas native DNA and RNA polymerase, separately, are readily filterable through Millipore membrane filters, a complex of the two is quantitatively retained by the filter. With this technique we have measured the stoichiometry and some properties of the complex between native T7 DNA and RNA polymerase.

Other polynucleotides, if added to RNA polymerase before but not after the T7 DNA, block formation of the T7 DNA–RNA polymerase complex. Measurements of the effect of polymer concentration on the extent of inhibition of complex formation between T7 DNA and RNA polymerase suggest that the number of binding sites at which RNA polymerase may bind to nucleic acids depends upon the primary and secondary structure of the polynucleotides.

2. Materials and Methods

(a) *Materials*

RNA polymerase, fraction IV (Chamberlin & Berg, 1962) was purified further by sedimentation through a preformed sucrose gradient. The specific activity, when assayed as previously described, was 3000 to 4000. *Escherichia coli* tyrosyl RNA synthetase was prepared according to Calendar & Berg (1966). T7 phage DNA was isolated and characterized (Jones, Dieckmann & Berg, manuscript in preparation) and denatured by incubation for 10 min in 0·1 M-NaOH; the pH was then adjusted to 7·5 to 8·0 with HCl. A consistent increase of 40 to 50% in the A_{260} accompanied denaturation. DNA from phage λ was prepared by a modified method of Kaiser & Hogness (1960), dAT was made according to Schachman, Adler, Radding, Lehman & Kornberg (1960) and poly dA and dT were gifts from Dr F. Bollum.

(b) *Methods*

The standard assay carried out for this work involved filtration of the putative RNA polymerase–DNA complex through a Millipore filter (type HA, plain, white, 2·4 cm diameter). The filters were soaked prior to use at room temperature for at least 10 min in 0·01 M-Tris, pH 8·0, containing 0·05 M-NaCl. The appropriate DNA and varying quantities of RNA polymerase were incubated (see legends to Figures for particulars conditions and variations on the basic experimental design), diluted with 2 ml. of the ice-cold 0·01 M-Tris–salt solution, immediately filtered through the membrane and the filter was washed with approximately 40 ml. of the same solution. With gentle suction, counting efficiencies of [^{3}H]DNA adsorbed on the filter were higher and more uniform than with strong suction. Filters were dried and counted with a toluene–POP–POPOP scintillation solution in a Nuclear-Chicago liquid scintillation spectrometer.

3. Results

(a) *Evidence for non-dissociable binding of RNA polymerase to DNA*

When RNA polymerase is added to a mixture of dAT and T7 DNA, RNA synthesis directed by the T7 DNA (GMP incorporation measures only the T7 RNA synthesis)

is inhibited compared to the reaction without dAT (Table 1). If the enzyme is added to T7 DNA one or two minutes before dAT is introduced, no inhibition of RNA synthesis is seen; however, if dAT is mixed with the enzyme before T7 DNA is added very little transcription of the T7 DNA ensues (Table 1).

TABLE 1

Inhibition of T7 DNA transcription by dAT as a function of when dAT is added to the reaction

DNA's added at 0 min	DNA's added at 2 min	GMP incorporation (mμmoles/20 min)
T7	—	4·4
dAT	—	<0·05
T7 + dAT	—	1·1
T7	dAT	4·4
dAT	T7	0·2

The reaction mixtures (0·3 ml.) contained 20 μmoles of Tris buffer, pH 7·4, 2 μmoles of $MgCl_2$, 1 μmole of 2-mercaptoethanol, 100 mμmoles of ATP, CTP, UTP and [^{32}P]GTP ($1·8 \times 10^6$ cts/min/μmole), 8 μg of RNA polymerase, and where indicated, 9 μg of T7 DNA and 4 μg of dAT. After 20 min at 37°C the nucleic acid was precipitated with acid, filtered through Whatman GF/C filters and counted.

Similarly, rAU synthesis with dAT as template (only rAU synthesis can occur with ATP and UTP as substrates) is inhibited by the presence of denatured T2 DNA (about 60% with the concentrations of dAT and denatured T2 DNA used here) but if dAT and enzyme are mixed first, added denatured DNA produces no inhibition (Table 2). The reverse order of mixing produces virtually complete inhibition of dAT copying. The same experiment done with native T2 DNA (using an amount equivalent to the denatured T2 DNA) shows that the helical polymer produces little or no inhibition of dAT copying when present from time zero and

TABLE 2

Inhibition of dAT transcription by native and denatured DNA as a function of when the DNA is added to the reaction

DNA added at 0 min	DNA added at 2 min	AMP incorporation (mμmoles/20 min)
dAT	—	6·6
Native or denatured T2	—	0·1
dAT + denatured T2	—	2·5
dAT	denatured T2	6·2
Denatured T2	dAT	0·2
dAT + native T2	—	6·4
dAT	native T2	6·6
Native T2	dAT	2·9

The reaction mixture was as in Table 1 except that the nucleoside triphosphates present were only UTP and [^{14}C]ATP (100 mμmoles of each) and where indicated 6 μg of native or denatured T2 DNA or 4 μg of dAT.

only about 50% inhibition when it is added before the dAT. These experiments indicate that the helical DNA is less efficient (on a nucleotide basis) than the single-stranded polymer as inhibitor of RNA polymerase.

(b) *Retention of native T7 DNA on membrane filters in the presence of RNA polymerase*

To examine further the characteristics of the binding between RNA polymerase and DNA, we employed a rapid and simple method for measuring this type of interaction. Native T7 DNA, as previously reported (Nygaard & Hall, 1963), readily passes through a Millipore membrane filter (>98%). In the presence of RNA polymerase, however, the DNA is retained by the filter. The amount of T7 DNA retained is proportional to the amount of RNA polymerase added (Fig. 1); with 2 μg of native T7 DNA the fraction retained is linearly dependent upon the RNA polymerase level up to 1 μg of enzyme (greater than 90% retention). With bovine plasma albumin (RNA polymerase diluent; Chamberlin & Berg, 1962), purified tyrosyl RNA synthetase or heat-denatured RNA polymerase, there is no retention of the T7 DNA (Fig. 1). The amount of labeled T7 DNA retained on filters under these conditions is not affected by the presence or absence of magnesium and 2-mercaptoethanol in the reaction mixtures.

If RNA polymerase is passed through the membrane first, followed by the ^{3}H-labeled T7 DNA, there is negligible retention of DNA. If the same concentration of both polymerase and DNA are mixed before filtration, complete retention of DNA occurs as shown in Fig. 1. This suggests that a complex between RNA polymerase and T7 DNA is formed on mixing the two and this complex is not filterable through Millipore membrane filters.

Although 1 μg of RNA polymerase is sufficient to cause retention of 2 μg of T7 DNA, this does not tell us how much enzyme can be bound to the DNA. To determine this, a fixed quantity of T7 DNA (2 μg) was mixed with increasing levels of enzyme (1 to 18 μg) under the conditions described in Materials and Methods and the legend to Fig. 1. With up to 5 μg of enzyme, no RNA polymerase activity was detected

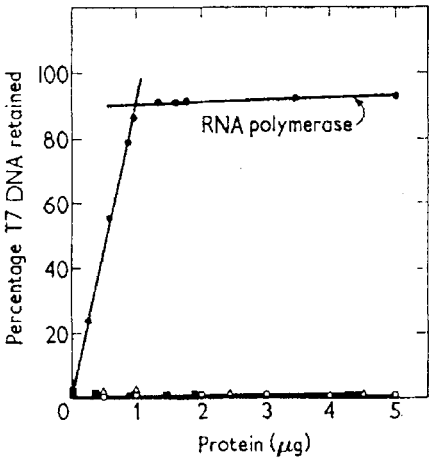

FIG. 1. RNA polymerase-dependent retention of ^{3}H-labeled T7 DNA on membrane filters. Reaction mixtures in a final volume of 0·25 ml. contained 2 μg of ^{3}H-labeled T7 DNA (approximately 14,500 cts/min), 10 μmoles of Tris–HCl buffer, pH 8·0, 1 μmole $MgCl_2$ and 3 μmoles of 2-mercaptoethanol. Incubation was for 5 min at 37°C and the amount of [^{3}H]DNA retained on the membrane was determined as described in Materials and Methods. RNA polymerase was denatured by heating at 70°C for 5 min. (△) Tyrosyl RNA synthetase; (■) denatured RNA polymerase; (○) bovine plasma albumin.

in the filtrate. With 7 μg of enzyme approximately 10% of the added activity appeared in the filtrate. With 9 and 12 μg of RNA polymerase added 2·8 and 6 μg, respectively, passed through the filter. Thus, all the RNA polymerase is bound to the T7 DNA until the weight ratio of enzyme to DNA reaches 3·0; when this ratio is exceeded unbound enzyme appears in the filtrate. If we assume that the molecular weight ratio of RNA polymerase to T7 DNA is between 0·02 to 0·04 (accepting the molecular weight of T7 DNA as 27×10^6 (Studier, 1965)) and of RNA polymerase as 0·5 to $1{\cdot}0\times10^6$ (Crawford *et al.*, 1965) and that enzyme with a specific activity of 3500 is approximately 50% pure, then at saturation of the T7 DNA there is between 35 to 70 moles of enzyme per mole of DNA. This agrees closely with the value for T7 DNA determined by Richardson (personal communication and referred to in Crawford *et al.*, 1965) using sedimentation analysis as a measure of binding; a value of about seven RNA polymerase binding sites per polyoma viral DNA (molecular weight 3×10^6) was reported by Crawford *et al.* (1965).

(c) *Formation of dAT–RNA polymerase complex*

Mixing dAT with RNA polymerase also produces a non-filterable complex (Fig. 2). Maximum retention of the dAT occurs when the weight ratio of enzyme to polynucleotide is 10 or about 20 times that found for T7. This is consistent with earlier findings (Wood & Berg, 1964) and with experiments discussed later and suggests that dAT contains many more binding sites for RNA polymerase than does an equivalent amount of T7 DNA.

(d) *Competition between DNA's in complex formation with RNA polymerase*

If dAT or denatured T7 DNA is mixed with RNA polymerase several minutes before adding T7 DNA, no T7 DNA is retained by the filter even when the weight ratio of enzyme to T7 DNA is two or four times the amount needed without the competing DNA (Fig. 3). When native phage λ DNA is added there is considerably less inhibition of subsequent RNA polymerase–T7 DNA complex formation. This suggests that phage λ DNA is less effective in binding RNA polymerase than is dAT or denatured T7 DNA.

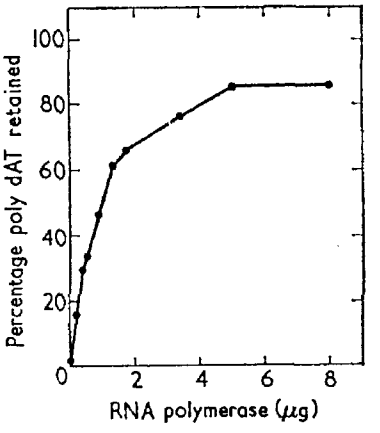

FIG. 2. Formation of complex between RNA polymerase and dAT and its retention on membrane filters.

Reaction mixtures were prepared as described in Fig. 1 except that 0·5 μg of [^{14}C]dAT (30,000 cts/min) was mixed with increasing quantities of RNA polymerase. All samples were treated and assayed as described in Fig. 1.

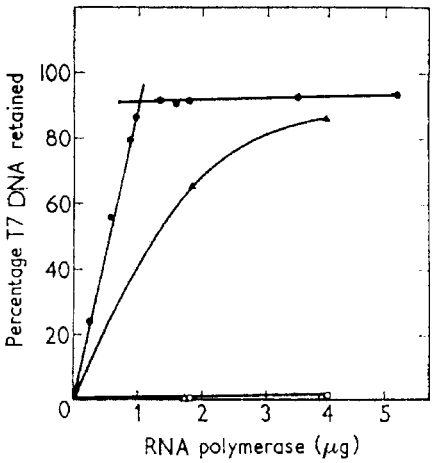

FIG. 3. Prevention of formation of RNA polymerase–T7 DNA complex by prior incubation of the enzyme with another DNA.

5 μg of λ DNA or 2 μg of denatured T7 DNA or poly dAT were added to separate reaction mixtures containing 2 or 4 μg of RNA polymerase. Each reaction mixture was incubated at 37°C for 3 min and then 2 μg of ^{3}H-labeled T7 DNA (15,000 cts/min) was added to each sample. After 2 min at 37°C each sample was diluted and filtered through Millipore filters as described in Materials and Methods. For comparison, a curve showing the extent of T7 DNA retention as a function of RNA polymerase in the absence of any other DNA is also shown. DNA added first: (●) none; (▲) λ DNA; (△) dAT; (○) denatured T7 DNA.

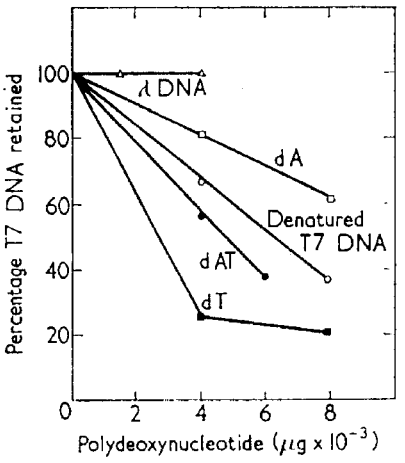

FIG. 4. Prevention of complex formation between RNA polymerase and T7 DNA by prior incubation of the enzyme with varying concentrations of other DNA's.

Reaction mixtures were prepared as described in Fig. 1, except that increasing concentrations of unlabeled DNA's were added to each reaction mixture containing 1 μg of RNA polymerase. After 3 min at 37°C, 2 μg of ^{3}H-labeled T7 DNA (13,000 cts/min) were added to each sample and after 2 min the samples were filtered as described in Materials and Methods. A control reaction mixture of ^{3}H-labeled T7 DNA and RNA polymerase alone was incubated.

A clearer demonstration of the capacity of different polymers to bind RNA polymerase is shown in Fig. 4. In this experiment, increasing quantities of several polynucleotides were mixed with 1 μg of RNA polymerase, before adding 2 μg of ^{3}H-labeled T7 DNA, and the amount of T7 retained on the filter was measured. Even at a weight ratio of enzyme to dAT or denatured T7 DNA of about 200 there is still approximately a 50% reduction in the amount of T7 DNA complexed to enzyme. By this test dA and dT are also effective in binding RNA polymerase; when added to the enzyme these polymers reduce the amount of enzyme available for binding to T7 DNA. At these low concentrations, phage λ DNA does not bind significant quantities of RNA polymerase, although as shown in Fig. 3, at higher concentrations of DNA, complex formation can be demonstrated.

(e) *Dissociation of RNA polymerase–DNA complex*

The RNA polymerase–T7 DNA complex is readily dissociated by raising the ionic strength (Fig. 5). Approximately 50% of the T7 DNA in the complex is liberated at salt concentrations between 0·15 and 0·20 M and essentially all the DNA is filterable at 0·3 M. In 0·4% sodium dodecyl sulfate, a concentration at which RNA polymerase is denatured, all of the DNA is filterable regardless of the salt concentration (Fig. 5). Comparable results on the dissociation of the RNA polymerase–DNA complex by sodium dodecyl sulfate and salt have been obtained using sucrose-gradient centrifugation to monitor the complex (Jones, Dieckmann & Berg, unpublished observations).

Can one polynucleotide replace another after the complex has been formed? The experiment in Table 1 indicates that within 20 minutes (the period of the assay), T7 DNA does not displace dAT from the complex with enzyme since little or no RNA synthesis occurs. Similarly, Table 2 shows that dAT is not copied when the RNA polymerase was first complexed to denatured T2; however, there could have been some dissociation of enzyme from the complex with native T2 DNA.

This question was examined in a study of the dissociation of the RNA polymerase–T7 DNA complex (measured as a decrease in the amount of ^{3}H-labeled T7 DNA

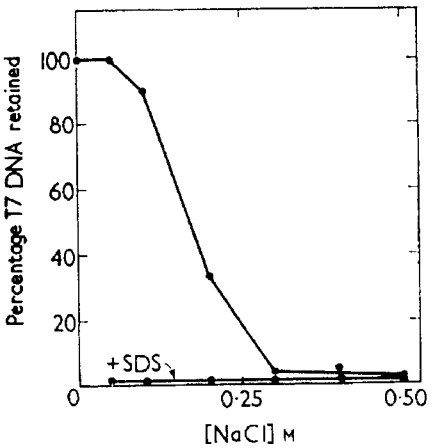

FIG. 5. Dissociation of T7 DNA from RNA polymerase–T7 DNA complex by salt or sodium dodecyl sulfate (SDS).

Complex was formed as described in Fig. 1 and either made 0·4% in SDS for 3 min at 37°C before dilution or diluted into 0·01 M-Tris, pH 8·0, containing increasing concentrations of NaCl. Filtration was as described in Materials and Methods.

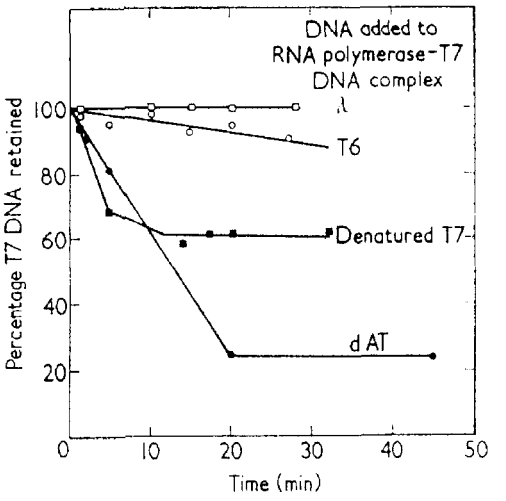

FIG. 6. Dissociation of RNA polymerase–T7 DNA complex by other RNA's.

Reaction mixtures were as described in Fig. 1, with a weight ratio of RNA polymerase to ^{3}H-labeled T7 DNA of 0·5 (the ratio at which all the DNA is retained on the filter). After 5 min at 37°C, an excess (2 to 3 times the amount of T7 DNA added initially) of a second polynucleotide was added to the mixture. Portions were removed for analysis by filtration at different times of incubation (37°C).

retained on the filter) by other polynucleotides (Fig. 6). The RNA polymerase–T7 DNA complex is quite stable at 37°C for at least an hour. If native λ or T6 DNA are added there is little or no release of T7 DNA from the complex after 30 minutes. If denatured T7 DNA or dAT is added to the complex, however, there is a time-dependent release of T7 DNA. Denatured T7 DNA releases about 40 to 50% of the native ^{3}H-labeled T7 DNA from the complex while dAT releases about 80% of the complexed ^{3}H-labeled T7 DNA. The reason for only partial dissociation of the T7 DNA from the complex is not known and is being investigated further. What is clear, however, is that, in general, dAT and denatured DNA form very strong complexes with RNA polymerase and apparently they can partially displace another DNA from the complex.

4. Discussion

The formation of complexes between RNA polymerase and ribo- or deoxypolynucleotides has been detected in different ways. RNA polymerase often occurs in crude extracts complexed to the endogenous DNA (Weiss, 1960; Hurwitz & August, 1963; Kadoya *et al.*, 1964); if purified RNA polymerase and a variety of polynucleotides are mixed and centrifuged through a sucrose gradient the enzyme and the nucleic acid sediment together (Hurwitz & August, 1963; Kadoya *et al.*, 1964; Bremer & Konrad, 1964; Fox *et al.*, 1965; Richardson, personal communication; Crawford *et al.*, 1965; Krakow, 1966). Polynucleotides when added to RNA polymerase before the template (i.e., that polynucleotide which is to be copied)

inhibit transcription of the template, but if added after the template there is little or no inhibition of transcription (Tissières, Bourgeois & Gros, 1963; Fox *et al.*, 1964,1965; Wood & Berg, 1964). Interestingly, RNA polymerase *per se* can inhibit replication or degradation of DNA by DNA polymerase or exonucleases, respectively (Berg *et al.*, 1965), presumably by blocking the site on the DNA at which these enzymes initiate their action.

In the present work we show that whereas the enzyme and native DNA, separately, pass through cellulose nitrate membrane filters, the complex formed by mixing the two is not filterable. This fact provides an easy and rapid way of measuring the stoichiometry and stability of the complex as well as the competition between polymers for binding the enzyme. We do not know why the combination of native T7 DNA with RNA polymerase is retained by a membrane filter. It is probably not due to the formation of intermolecular aggregates of DNA molecules since under conditions where complete retention of DNA occurs (i.e., 1 μg of enzyme and 2 μg of T7 DNA, Fig. 1) there is no significant alteration of the sedimentation coefficient or profile of the DNA. Retention of the DNA on the filter is also probably not due to introduction of single-strand breaks or degradation of the DNA since if the mixture of enzyme and DNA is made 0·5 M in salt or if it is treated with 0·4% sodium dodecyl sulfate all the DNA becomes filterable. As pointed out by Nygaard & Hall (1963), denatured DNA is retained on membrane filters in 0·9 M-KCl. Moreover, the enzyme fraction used for these studies does not introduce detectable single-strand interruptions in T7 DNA or M13 DNA rings (Jones, Dieckmann & Bera, unpublished observations). Since RNA polymerase is readily filterable, it is not likely that the complex is retained because of some interaction between the enzyme and the filter. It is known that denatured DNA retained by nitrocellulose filters (Nygaard & Hall, 1963) and conceivably the binding of enzyme to the DNA produces a collapse of the helical structure over a localized region (where transcription will be initiated) thereby altering the rod-like structure of the DNA.

The amount of RNA polymerase bound per T7 DNA molecule is clearly not limited by a lack of space on the DNA surface (see Crawford *et al.*, 1965). It seems a reasonable inference that the binding sites are unique but the structural basis for the uniqueness is not known. We recently speculated (Berg *et al.*, 1965) that the binding sites resulted from interruptions in the interstrand base-pairing at specific but short regions in the DNA molecule. We assumed that the enzyme could only bind to unpaired regions and that these were generated only at unique sequences. Quite conceivably, if the binding sites were also the points of initiation of RNA synthesis, the strand selection might be made on the basis of the base sequence (pyrimidine content?) in this region. If the binding sites did represent boundaries for mRNA initiation and termination the average chain length of the 35 to 70 T7 mRNA's would be about 600 to 1200 nucleotides.

Although further study is necessary it is quite clear already that the nature of the RNA polymerase–polynucleotide complex is influenced by the secondary structure and nucleotide sequence of the nucleic acid. For example, denatured or single-stranded DNA and even dAT bind considerably more RNA polymerase than does native helical DNA. Since 0·006 μg of denatured DNA can bind 0·5 μg of RNA polymerase (Fig. 4) and approximately 0·15 μg of native DNA binds 0·5 μg of enzyme (see p. 202), it appears that denatured DNA binds about 25 times more polymerase than does helical DNA. dAT, in spite of its helical structure, resembles denatured

DNA in its binding capacity for RNA polymerase. This may result from the existence of a large number of unpaired regions due to "looping" and short hairpin-like structures (Inman & Baldwin, 1962; Spatz & Baldwin, 1965) present in the enzymically synthesized dAT, or to the generally weaker interstrand interactions characteristic of the A–T base pair (Sueoka, Marmur & Doty, 1959).

The marked difference between the "affinity" of λ DNA and T7 DNA for RNA polymerase is of interest but not yet understood. Our data suggest that DNA from λ phage contains far fewer (perhaps 5 to 10%) strong binding sites for RNA polymerase than does T7 phage DNA. This follows from the finding (Fig. 3) that 5 μg of λ DNA binds only 1·5 μg of RNA polymerase (i.e., an amount of enzyme capable of causing retention of 65% of the challenging dose (2 μg) of ^{3}H-labeled T7 DNA); an equivalent amount of T7 DNA would strongly bind approximately 30 μg of enzyme. Whether the few RNA polymerase binding sites on λ DNA are confined to the right half of the λ DNA molecule, the segment preferentially transcribed by RNA polymerase *in vitro* (Maitra, Cohen & Hurwitz, 1966; Gros & Naono, 1966), is not yet established.

Of the polynucleotides tested, dT is even more effective in complexing RNA polymerase than denatured DNA. Whether this is due only to its secondary structure or whether the sequence of thymidine residues *per se* influences the number of binding sites for RNA polymerase needs further study. It is of interest that dT appears to bind about four times more enzyme than does dA. It should be pointed out that RNA synthesis *in vitro* most frequently starts with a purine residue (Bremer *et al.*, 1965; Maitra & Hurwitz, 1965), that is, copying *in vitro* is initiated by a pyrimidine in the template. Moreover, poly A synthesis catalyzed by RNA polymerase (Chamberlin & Berg, 1962,1964; Stevens, 1961) has been postulated to result from reiterative transcription of thymidine sequences in the template (Chamberlin & Berg, 1964). Perhaps the failure to observe extensive reiterative transcription with other single nucleoside triphosphates is related to the preferential binding of RNA polymerase to sequences of thymidine as well as to any differences in the chain initiation and elongation phases.

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